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rbt@cariari.ucr.ac.cr
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Chaves, Fernando; Chacón, Mauricio; Badilla, Beatriz; Arévalo, Carolina
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Universidad de Costa Rica
San Pedro de Montes de Oca, Costa Rica

Effect of *Echinacea purpurea* (Asteraceae) aqueous extract on antibody response to *Bothrops asper* venom and immune cell response

Fernando Chaves[1], Mauricio Chacón[2], Beatriz Badilla[2]* & Carolina Arévalo[2]

1 Instituto Clodomiro Picado, Facultad de Microbiologia, Universidad de Costa Rica, 2060, Costa Rica.

2 Instituto de Investigación Farmacéutica, INIFAR, Facultad de Farmacia, Universidad de Costa Rica, 2060, Costa Rica; * bbadilla@cariari.ucr.ac.cr

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Abstract: The effect of aqueous extract of *Echinacea purpurea* roots on the murine antibody response to *Bothrops asper* snake venom *in vivo* was studied. Three groups were used. Group #1, baseline control, was treated with snake venom plus PBS. Group #2 was treated with snake venom plus sodium alginate as adjuvant (routine method used at Instituto Clodomiro Picado), and group #3 or experimental group, was treated with snake venom plus aqueous extract of *E. purpurea* root as adjuvant. In all groups, the first inoculation was done with Freund's complete adjuvant (FCA). By the time of the second bleeding, mice in group #3 showed a remarkable increment in the level of anti-venom antibodies compared with those in groups #1 or #2. *In vitro* immune cell proliferation as a response to aqueous extract of *E. purpurea* root was studied using human lymphocytes activated with different lectins (Con A, PHA and PWM). In all cases, increase in percentage of lymphoproliferation was greater when *E. purpurea* root extract was used in addition to individual lectins. Rev. Biol. Trop. 55 (1): 113-119. Epub 2007 March. 31.

Key words: *Echinacea purpurea,* immunostimulatory properties, adjuvant, plant extract, *in vitro* lymphoproliferation, murine antivenom antibodies.

Echinacea purpurea root is commonly used around the world for stimulation of immune system (Redondo 2000). It is used as herbal medicine in respiratory infections, against malignant tumors and several inflammatory conditions (Elsasser-Beile *et al.* 1996, Hill *et al.* 1996, Burger *et al.* 1997, Percival 2000, Redondo 2000). Some of the active compounds of *Echinacea* have been identified, such as alkylamides, chicoric acid, arabinogalactan, glycoproteins and polysaccharides. The proportions of these components differ depending on the part of the plant analyzed (Bodinet *et al*. 1993, Facino *et al.* 1995, Redondo 2000).

Different researchers have shown that this plant can be used as enhancer of the immune response, specially of cells such as neutrophils, monocytes and lymphocytes (Stotzem *et al.* 1992, Schoneberger 1992, Bodinet *et al.* 1993, Miller 1998). Rehman *et al.* (1999), and Bodinet and Freudenstein (1999) have shown that extracts of *Echinacea* are able to increase the titer of specific antibodies against antigens, such as keyhole hemocyanin and sheep red blood cells, in the sera of treated animals. Redondo (2000) proposes that this plant stimulates the acquired immune system because of its specific action on lymphocytes, enhancing the production of antibodies and T cell responses. Other investigators have indicated that root extracts from *Echinacea* are able to stimulate the production of cytokines, such as IL-1, IL-10 and TNF-α by human macrophages (Burger *et al.* 1997).

At Instituto Clodomiro Picado, we use the venom of *Bothrops asper*, together with, those

of *Crotalus durissus* and *Lachesis stenophrys*, as an immunization mixture in the production of horse polyvalent antiserum for the treatment of pit viper bites (Bolaños and Cerdas 1980, Gutiérrez *et al.* 1988, Gutiérrez 2002). *B. asper* (commonly known as "*terciopelo*") is the most important venomous snake in Central America from the standpoint of human mortality and morbidity causing the majority of envenomations in these countries (Bolaños 1982, Campbell and Lamar 1989, Hardy 1994, Rojas *et al*. 1997, Arroyo *et al.* 1999, Sasa 2002).

In this work, we investigated the use of *E. purpurea* root extract to improve the *in vivo* anti-venom humoral immune response of mice immunized with *B. asper* venom. In addition, we studied the *in vitro* effect of this extract on the mitogenic response of human lymphocytes to different lectins (PHA, PWM and Con A).

MATERIALS AND METHODS

Snake venom: it was obtained from adult specimens of *B. asper* collected in the Pacific region of Costa Rica. Venom pools were made from more than 40 individual snakes. After lyophilization, the venom was maintained at -20 ºC. This process is routinarily used at Instituto Clodomiro Picado, Universidad de Costa Rica.

Plant extract: crude powder of *E. purpurea* root was kindly provided by "Industria Los Patitos, S.A.", Heredia, Costa Rica. A 10% aqueous extract was prepared by infusion with 70 °C water for 30 min. This extract was filtered, vacuum concentrated, lyophilized and kept in dark bottles at 4 °C until used.

Experimental animals: female White Swiss Webster mice (18-20 gm), supplied by Instituto Clodomiro Picado were used.

Human white blood cells: peripheral blood mononuclear cells (PBMC)) were obtained from anti-coagulated human blood using a Ficoll-Hypaque (Sigma, St. Louis, MO) gradient. In the case of lymphoproliferation experiments, human blood from young male volunteers 20-25 years old, was used.

Immunization protocol: for murine experiments, the immunization protocol used was based on the one described by Rucavado *et al*. (1996), with slight modifications. Three groups were established: Group #1 (baseline control) treated with snake venom plus 0.1 M phosphate buffered saline pH 7.2 (PBS). Group #2 was treated with snake venom plus sodium alginate as adjuvant (routinary method used at Clodomiro Picado). Group #3, experimental group, was treated with snake venom plus aqueous extract of *Echinacea purpurea* root as adjuvant. During the first immunization, all groups received a subcutaneous (sc) injection of venom (20 µg in 100 µl PBS), emulsified in an equal volume of Freund's Complete Adjuvant (FCA). Following the first immunization, at two week intervals, each group received sc injections of increasing amounts of venom (20, 40 and 60 µg each diluted in 100 µl PBS) using as adjuvants either an equal amount of sodium alginate or *Echinacea* root extract (groups #2 or #3 respectively). In group #1 (baseline control), the first venom dose was emulsified with FCA. The rest of immunizations were administered with PBS alone (no adjuvant).

After the fourth immunization, mice were tail bled at two weeks intervals. One week after each bleeding, additional injections of 40 µg venom, with the corresponding adjuvant, were performed. From the first immunization until the first bleeding, group #3, was injected with 100 µg of *Echinacea* extract. Thereafter, *Echinacea* dose was increased to 200 µg of extract.

Enzyme linked immunosorbent assay (ELISA): the change in antibody levels was measured by enzyme linked immunosorbent assay (ELISA). 100 µl of venom suspension (1 µg/100 µl in PBS with 2% BSA) were placed in each well of 96 well plates (Dynatech, Immulon II) and incubated overnight at 4 ºC. Plates were washed three times with PBS immediately before use. A 1/2000 dilution of each serum was added by triplicate, incubated at room temperature for one hour and washed as described before. Normal mouse serum was used as control. Bound antibodies were

detected using an anti-mouse-IgG antiserum peroxidase-conjugated (Sigma). O-phenylenediamine (Sigma) was used as substrate, color development was directly proportional to IgG concentration. Absorbances were recorded at 492 nm (Dynatech 500).

Mitogenic response of human lymphocytes: lymphocytes were obtained from anticoagulated human blood using a Ficoll-Hypaque (Sigma, St. Louis, Mo.) gradient. After washing twice with RPMI (GIBCO), the cells were resuspended to 5 X 10^6 cells/ml in RPMI plus 10% fetal calf serum (FCS), 100 µl of this cell suspension were placed in each well.

Lymphocytes were stimulated with aqueous extract of *E. purpurea* at different final concentrations. Three lectins (PHA, Con A, PWM) in three different concentrations each, were tested separately as costimulant molecules required for this assay. PHA final well concentrations were: 0.5, 5.0 and 10.0 µg/ml. PWM final well concentrations were 2.5, 5.0 and 10.0 ng/ml. Con A final well concentrations were 2.5, 5.0 and 10.0 µg/ml. Final concentration of *Echinacea* extract was also varied using serial dilutions (final well concentrations: 0; 4; 8; 16; 33; 66; 133; 266 µg/ml). Lymphocytes in the presence of *Echinacea* extract and lectin were incubated during four days at 37 °C, 5% CO_2, 80% humidity in a Cole Parmer CO_2 incubator. After incubation, 25 µl of 3-[4-5-dimethylthiazol-2-yl] 2,5 diphenyl-tetrazolium bromide (MTT) (Sigma, St. Louis, MO) were added to each well followed by 4 additional hours of incubation at 37 °C. Reaction was stopped adding 50 µl SDS 20% in dimethylformamide pH 4.7. Absorbances were recorded at 550 nm (Biorad Microplate Reader Model 550).

Percentage of proliferation obtained with lectins alone (no addition of *Echinacea* extract), was established as 100% and used as experimental control. Percentage of proliferation obtained with lectin plus *Echinacea* extract was calculated by dividing the absorbance of the experimental well (lectin plus *Echinacea*) by the absorbance of the control well (lectin alone) and multiplying by 100. If the percentage obtained was significantly higher than 100%, the extract was considered stimulatory. Likewise, if it was significantly lower than 100%, it was considered inhibitory of lymphocyte proliferation.

Statistical analyses: results are presented as the mean ± standard deviation. Statistical significance of the differences in mean absorbances between experimental groups was performed by one-way analysis of variance, and the significant differences ($p < 0.05$) were compared by Tukey-Kramer multiple comparison test.

RESULTS

Antibody production: antibody levels corresponding to first bleeding (two weeks after 4th immunization) and second bleeding (four weeks after 4th immunization) are shown in Figure 1. At the moment of the first bleeding, mice in group #3 (venom plus *E. purpurea* root extract), had a slightly higher level of antibodies (no significant difference) than mice in Groups #1 or #2.

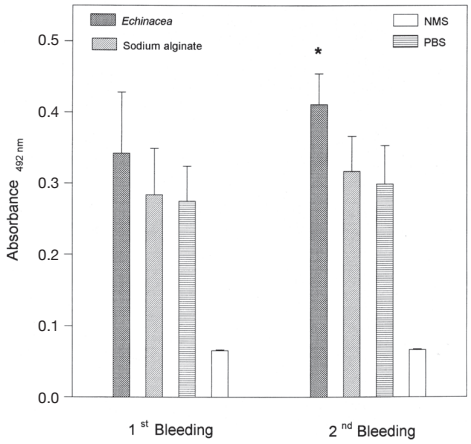

Fig. 1. Specific anti-venom antibody levels in sera of mice injected with *Bothrops asper* venom and different adjuvants. Mice receiving injections of venom plus *Echinacea* extract (Echinacea) showed higher levels of specific antibody than mice injected with venom plus sodium alginate (Sodium alginate) or PBS (PBS). Baseline levels of antibody in normal mice sera are shown (NMS). Results are shown as mean + SD of absorbances obtained from the ELISA test (*$p<0.05$).

At the time of the second bleeding, group #3 developed a higher antibody titer (a statistically significant), compared to the other groups tested (Fig. 1). Group #2, did not improve antibody levels, compared to mice in group #1 (Fig. 1).

Lymphocyte mitogenic response: Stimulation of human lymphocyte cultures with PWM and *Echinacea* shows an increase in cell proliferation at PWM concentrations of 2.5 ng/ml and 10 ng/ml (250% and 150% respectively) (Fig. 2), compared to growth of lymphocytes with PWM and no *Echinacea* extract. Using PWM 5 ng/ml there was no significant change in level of proliferation.

In lymphoproliferation assays using PHA plus *Echinacea*, we observed an increase in lymphocyte proliferation at all of the lectin concentrations used (0.5; 5.0 and 10 µg/ml). The best stimulation (431%) was achieved with 10 µg/ml of PHA at 4 µg/ml of *Echinacea* extract (Fig. 3). Using PHA 5 µg/ml and *Echinacea* extract at 4 µg/ml, we obtained 282% of stimulation. With 0.5 µg/ml of PHA and 66 µg/ml of *Echinacea* extract, we obtained 215% stimulation (Fig. 3).

Concerning experiments with Con A and *Echinacea* extract (Fig. 4), percentage of cell growth was slightly stimulated (158%) at Con A concentrations of 2.5 µg/ml and *Echinacea* 34 µg/ml. At Con A 5.0 µg/ml and *Echinacea* 16 µg/ml, a slight but not significant (128%) increase was observed. At 10 µg/ml of Con A, there was a decrease in percentage of proliferation up to 28% at an *Echinacea* concentration of 66 µg/ml.

Figure 5 shows results of the highest lymphoproliferation obtained with each one of the three different lectins. The lectin concentrations shown are PWM 2,5 ng/ml, PHA 10 µg/ml and Con A 10 µg/ml. In all cases, best results were obtained at concentrations of *Echinacea* lower than 100 µg/ml. At higher *Echinacea* concentrations the lymphoproliferation response was milder or nule. PHA increased lymphocyte proliferation up to approximately 400% and PWM stimulated lymphochyte proliferation up to approximately 250%.

DISCUSSION

In studies accomplished by Rucavado *et al*. (1996) the adjuvant properties of various immunostimulants (sodium alginate, calcium alginate, aluminum hydroxide and muramyl

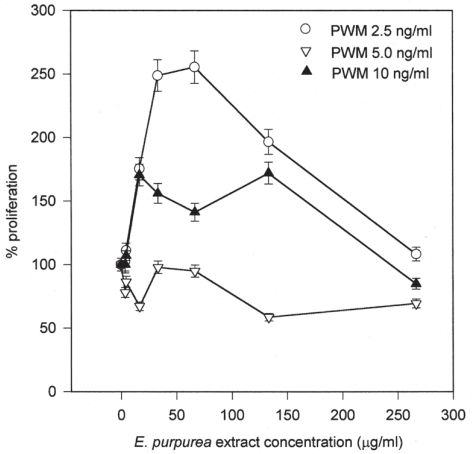

Fig. 2. Effect of aqueous extract of *Echinacea purpurea* plus PWM on lymphoproliferation *in vivo*. Human lymphocytes were cultured in the presence of PWM (2.5, 5.0 and 10 ng/ml respectively) and *Echinacea purpurea* root extract. Results are shown as mean + SD.

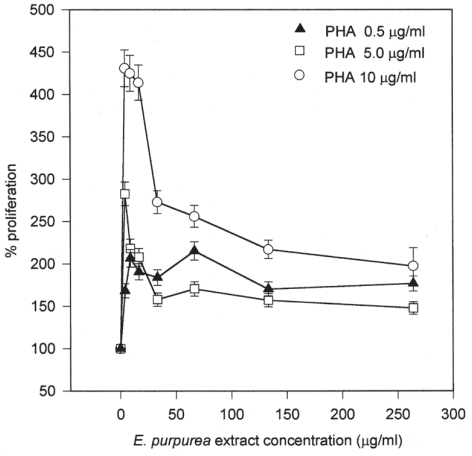

Fig. 3. Effect of aqueous extract of *Echinacea purpurea* plus PHA on lymphoproliferation *in vivo*. Human lymphocytes were cultured in the presence of PHA (0.5, 5.0 and 10.0 µg/ml respectively) and *Echinacea purpurea* root extract. Results are shown as mean + SD.

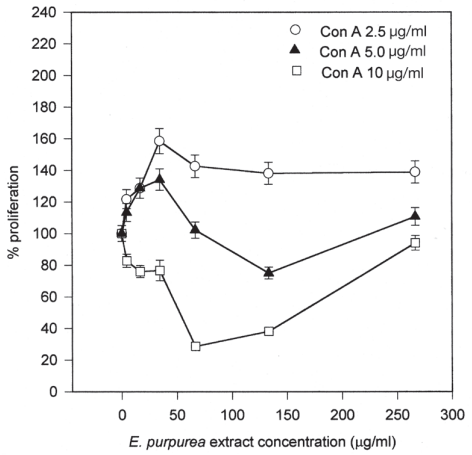

Fig. 4. Effect of aqueous extract of *Echinacea purpurea* plus Concanavalin A on lymphoproliferation *in vivo.* Human lymphocytes were cultured in the presence of Con A (2.5, 5.0 and 10.0 µg/ml respectively) and *Echinacea purpurea* root extract. Results are shown as mean + SD.

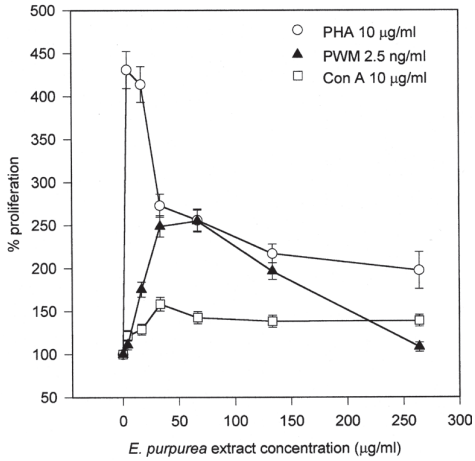

Fig. 5. Summary of effects of aqueous extract of *Echinacea purpurea* plus different lectins on lymphoproliferation *in vivo.* Human lymphocytes were cultured in the presence of different lectins (Con A 10 µg/ml, PHA 10 µg/ml or PWM 2.5 ng/ml) and *Echinacea purpurea* root extract. Results are shown as mean + SD.

dipeptide) on murine antibody response against *B. asper* were evaluated. They found that the level of immunopotentiation obtained by all of these molecules was very similar. The purpose of this research was to determine if the aqueous extract of *E. purpurea* roots had adjuvant properties on immune response to snake poisons, which might be applied in the industrial production of anti-venom antiserum.

Researchers have shown that extracts from different parts of *Echinacea* sp. are able to increase both specific and non-specific immune response, such as activation of neutrophils, macrophages, T and B cells (Stotzem *et al*. 1992, Bodinet *et al*. 1993, Facino *et al.* 1995, Rehman *et al.* 1999, Bodinet and Freudenstein 1999). See *et al*. (1997) and Percival (2000) suggest that *Echinacea* affects only the phagocytic immune system, specifically acting on macrophages and neutrophils. It is possible that there are different costimulatory molecules present in *Echinacea* extracts tested (Bodinet *et al.* 1993, Redondo 2000). We suggest that B cell activation is due to the presence of polysaccharides, such as arabinolactanes, which act as polyclonal activators.

In this work, we demonstrate that the aqueous extract of *Echinacea* roots stimulates proliferation of human lymphocytes *in vitro* and is able to increase specific antibody response *in vivo*. We show that *E. purpurea* root extract is 30% more potent than sodium alginate with respect to stimulation of anti-venom antibodies, and it can be used together with other adjuvants (e.g. Freund´s).

Results indicate that there is a conspicuous difference in the anti-venom antibody levels in mice treated with *Echinacea* compared with mice treated with PBS or sodium alginate alone. During the first stage of the immunization schedule, the difference between antibody levels of mice treated with *Echinacea* compared to the other experimental groups was very slight. After the second bleeding, the immune response of mice injected with *Echinacea* reached much higher levels than the other two groups.

We performed the *in vitro* lymphoproliferation assays in order to test if *Echinacea* extract was acting directly as a mitogen of immune cells. By using the different lectins we were trying to determine if *Echinacea*

root extract was acting preferentially on a specific population of lymphocytes (T or B cells). PWM is known as a T and B cell mitogen while PHA and Con A are mitogens for T cells. This selectivity is due to the binding of lectins to different sugar radicals on the cell surface. In our experiments, we observed that PHA and PWM stimulated human lymphocyte proliferation *in vitro*, at concentrations of *Echinacea* root extract lower than 100 µg/ml. We propose that *Echinacea* root extract is activating proliferation of T and B cells. The fact that the *Echinacea/*Con A combination did not improve lymphoproliferation in a significant manner compared to *Echinacea*/PHA could be due to the effect of this lectin on a different subpopulation of T cells. This effect could also be due to toxicity of the *Echinacea/*Con A combination at these specific concentrations. We believe that one or several components present in the plant extract, act as costimulatory molecules, potentiating the mitogenic effect induced by PHA or PWM.

Some researchers have shown that *Echinacea* extracts are able to promote the production of cytokines such as IL-1, IL-2, TNF, IL-6 and INF-γ (Elsasser-Beile *et al.* 1996, Burger *et al.* 1997). This ability of *Echinacea* might contribute to improve the *in vivo* immune response observed in our experiments.

In conclusion, our observations indicate that the aqueous extract of *Echinacea* is able to stimulate *in vivo* the production of antibodies against the specific antigen *B. asper* venom and to stimulate in vitro the proliferation of lymphocytes. We suggest that this extract should be used as immunostimulant in the immunization protocols of animals such as horses during production of antisera (e.g. antiofidic sera).

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RESUMEN

Se estudió *in vivo,* el efecto del extracto acuoso de las raíces de *Echinacea purpurea* en la respuesta de los anticuerpos murinos al veneno de la serpiente *Bothrops asper*. El grupo 1 control, fue tratado con el veneno y PBS. El grupo 2 con veneno y alginato de sodio (método utilizado en el Instituto Clodomiro Picado), y el grupo 3 o experimental, con veneno y extracto acuoso de las raíces de *E. purpurea*. En todos los grupos, la primera inmunización fue hecha con FCA (Freund's Complete Adjuvant). En las muestras correspondientes a la segunda sangría, los ratones del grupo 3 mostraron un marcado incremento en el nivel de anticuerpos, en comparación con los ratones de los otros grupos. También se determinó la proliferación de células inmunes *in vitro,* como respuesta al extracto acuoso de la raíz de *E. purpurea,* utilizando linfocitos humanos activados con diferentes lectinas (Con A, PHA y PWM). En todos los casos, el incremento en el porcentaje de linfoproliferación fue mayor, cuando estaba presente el extracto de la raíz de *E. purpurea*.

Palabras clave: *Echinacea purpurea,* propiedades inmunoestimulatorias, extracto de plantas, linfoproliferación *in vitro*, anticuerpos de anti-veneno murino.

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